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Human Leukocyte Antigen-B-Associated Transcript 3 Is Released from Tumor Cells and Engages the NKp30 Receptor on Natural Killer Cells

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SUMMARY

The activity of natural killer (NK) cells is regulated by surface receptors, which direct target cell recognition. NKp30 (Natural Cytotoxicity Receptor 3) induces target cell lysis and is also crucial for the interaction with dendritic cells. So far, the cellular ligands for NKp30 have remained elusive. Here we show that the nuclear factor HLA-B-associated transcript 3 (BAT3) was released from tumor cells, bound directly to NKp30, and engaged NKp30 on NK cells. BAT3 triggered NKp30-mediated cytotoxicity and was necessary for tumor rejection in a multiple myeloma model. These data identify BAT3 as a cellular ligand for NKp30. We propose a concept for target cell recognition by NK cells beyond "missing self" and "induced self," mediated through a tumor cell-derived extracellular factor.

INTRODUCTION

Natural killer (NK) cells are lymphocytes of the innate immune system, which directly attack tumor and virus-infected cells. They provide a link between innate and adaptive immunity through crosstalk with dendritic cells (DCs) (Moretta et al., 2007; Munz et al., 2005; Walzer et al., 2005) and mediate T cell activation (Adam et al., 2005; Hanna et al., 2004; Laouar et al., 2005). The natural cytotoxicity receptors (NCR) including NKp30, NKp44, and NKp46 as well as NKG2D are triggering receptors responsible for NK cell activation (Lanier, 2005; Moretta and Moretta, 2004). Activating receptors and their corresponding ligands play a vital role for tumor cell recognition and tumor surveillance. A decreased expression of ligands engaging NCR and NKG2D on tumor cells correlating with impaired tumor recognition and tumor progression has been reported for both hematological malignancies such as multiple myeloma (Carbone et al., 2005) and solid tumors (Moretta et al., 2006). The engagement of NKG2D, moreover, inhibits the formation of chemically induced sarcomas (Smyth et al., 2005) and the experimental overexpression of NKG2D-specific ligands on tumor cells leads to tumor rejection and immunity (Cerwenka et al., 2001; Diefenbach et al., 2001; Friese et al., 2003). Until now, little was known about the molecular nature of the cellular ligands for NKp30, NKp44, and NKp46. These molecules are so far identified only indirectly by receptor-specific antibodies that inhibit the NK cell cytotoxicity against target cells (Bottino et al., 2005; Farag and Caligiuri, 2006; Pende et al., 1999).

The orphan NKp30 receptor (NCR3, CD337) plays a special role because it is the only receptor involved in both tumor cell lysis and lysis of normal self cells (Ferlazzo et al., 2002; Pende et al., 1999; Vitale et al., 2005). The 30 kD triggering receptor is selectively expressed on NK cells and associated with CD3z chains that become phosphorylated upon ligation (Pende et al., 1999). Human dendritic cells (DCs) express the hitherto unknown NKp30 ligand that mediates the NK-DC interaction, resulting either in DC activation or DC killing, thus limiting the supply of dendritic cells (Ferlazzo et al., 2002; Pende et al., 1999; Vitale et al., 2005). Although the cellular ligands binding to the NCR receptors are not yet identified, functional data indicate that they are expressed on cells upon activation, proliferation, or tumor transformation (Bottino et al., 2005). So far, it has been unclear why cell lines such as fibroblasts or lymphoma cells that are killed by NK cells in an NKp30-dependent manner fail to bind to soluble NKp30-Ig fusion proteins (Arnon et al., 2005; Bottino et al., 2005; Mandelboim et al., 2001; Pende et al., 1999). Therefore, we used a yeast two-hybrid approach to isolate a putative NKp30 ligand, which is probably not constitutively expressed on the cell surface. Here we show that the nuclear factor HLA-B-associated transcript 3 (BAT3) was released from tumor cell in response to stress signals and engaged NKp30 on NK cells. Recently it was discovered that nuclear BAT3 is responsible for the p53-mediated cellular response to stress and DNA damage, resulting either in DNA repair or in apoptosis, which ultimately suppresses tumor growth (Sasaki et al., 2007). BAT3 is thus a molecule, which directly links p53 tumor

suppressor function with a danger signal alerting the immune system via an activating receptor on NK cells.

RESULTS

BAT3 Is a Direct Binding Partner for NKp30

To identify proteins interacting with NKp30, a fusion construct consisting of the GAL4-DNA-binding domain and the extracellular NKp30 sequence (GBT9-NKp30) was used as bait. We screened a K562-derived cDNA library and isolated a clone encoding the C-terminal sequence of BAT3 (BAT3-CT). The cDNA fragment of 1.6 kb was characterized by a deletion of the highly conserved Bcl-2-associated athanogene (BAG) domain (see Figure S1A available online). This domain is responsible for the reported binding of BAT3 to Heat Shock Protein 70 (HSP70) (Takayama et al., 1997; Thress et al., 2001). Both isoforms, either including or lacking the BAG domain, are expressed in tumor tissues, cell lines, and monocyte-derived dendritic cells (Figure S1B). Analysis of BAT3 (full-length) and a panel of deletion constructs showed that the 1.6 kb C-terminal fragment was necessary and sufficient for the direct binding to NKp30, irrespective of the BAG domain. The NKp30 binding, however, may include a dimerization of BAT3 molecules, as indicated by the fact that a BAT3-BAT3 interaction was observed (Figure S1A). Several control constructs that failed to interact with BAT3 or NKp30, respectively, were used to prove the specificity of the two-hybrid results (noted in the legend to Figure S1A).

The direct and specific binding of NKp30 to BAT3 was confirmed by coimmunoprecipitation. Wild-type 293T cells (WT) and a 293T cell line that constitutively expressed the extracellular domain of NKp30 fused to the Fc sequence of human IgG1 (NKp30-Ig; Figure S1C) were transfected with BAT3-CT or BAT3 expression constructs encoding histidine-tagged proteins. Both BAT3 constructs were coprecipitated by isolation of NKp30-Ig from the transfected cells with protein-G sepharose that binds to Fc sequence (Figure 1). No unspecific precipitation was detectable in extracts of cells transfected with CD30-Ig (not shown), wild-type cells, or in cells overexpressing the control protein hepatocyte nuclear factor 4 (HNF4) (Figure 1, left lane). These experiments proved the specificity of the BAT3-NKp30 interaction in mammalian cells and further supported the idea that BAT3 is a putative ligand for NKp30.

The gene encoding human BAT3 was originally identified within the inflammatory class III region of the human major histocompatibility locus on chromosome 6 (Banerji et al., 1990). BAT3 is thus clustered with other immune regulatory genes such as NKp30, TNF, or LTA. A role for BAT3 in regulating both proliferation and cell death has been discussed. BAT3 seems to trigger ricin-induced apoptosis (Wu et al., 2004), and BAT3-deficient cells are more resistant to apoptosis in response to agents affecting the calcium flux in the endoplasmatic reticulum (Desmots et al., 2005) or genotoxic stress (Sasaki et al., 2007). Recently, a gene-depleting approach was used to show the precise function: nuclear BAT3 has an essential role in controlling the acetylation of p53, which is required for the cellular DNA damage response (Sasaki et al., 2007).

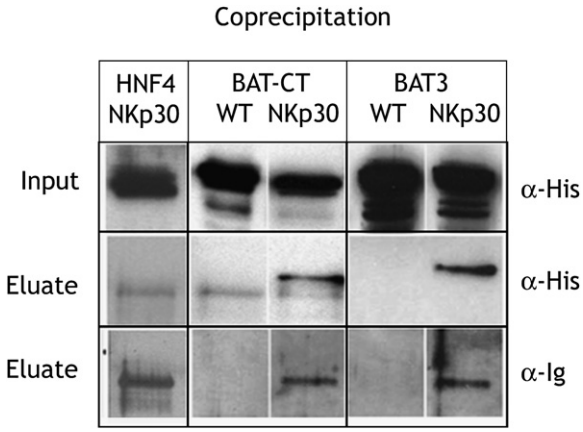

Figure 1. Coimmunoprecipitation of BAT3 and NKp30

Wild-type 293T cells (WT) or NKp30-Ig-expressing 293T cells (NKp30) were transfected with $(His)_6$HNF4 (control), $(His)_6$BAT3-CT (70 kda), or $(His)_6$BAT3 (>130 kda), and the lysates were analyzed for expression (a-His Blot, input). The lysates were used for immunoprecipitation with protein-G sepharose followed by immunoblotting of the eluates with monoclonal anti-his (a-His) or anti-Ig (a-Ig, eluate). In control experiments, no protein precipitation was detectable with 293T cells, which constitutively expressed CD30-Ig or upon transfection of $(His)_6$HNF4 expression vector (left lane).

BAT3 Is Released from Tumor Cells

Because a nuclear factor is not accessible for the interaction with the surface receptor NKp30, we tested directly whether BAT3 was released from tumor cells. 293T cells were exposed to a nonlethal heat shock and the BAT3 distribution was monitored in subcellular fractions and the cell supernatant (Figure 2A). Endogenous BAT3 was predominantly expressed in the nuclear fraction. However, it was also detectable in the membrane fractions and cell supernatants in response to a nonlethal heat shock (Figure 2A, left). Overexpressed BAT3 was, similarly to the endogenous protein, detectable in the nuclei. Upon heat shock, BAT3 was found in the membrane and released from the cells (Figure 2A, middle). No changes were observed for the distribution of the nuclear protein p53 (Figure 2A, right). The number of apoptotic or necrotic cells was not increased by heat shock (Figure S2C), thus excluding the possibility that apoptotic or necrotic cells were the source for BAT3.

Next, the expression pattern of BAT3 upon contact with NK cells was analyzed. As expected, a transfected histidine-tagged BAT3 protein ($(His)_6$BAT3) was mainly localized in the nuclei of 293T cells (Figures 2B and 2D, red staining). Upon coincubation with the human NK cell line NKL (Figure 2C, green staining) or primary NK cells (Figure 2E), this subcellular expression pattern changed dramatically. The nuclear BAT3 staining disappeared and instead staining of the cell membrane became evident. The

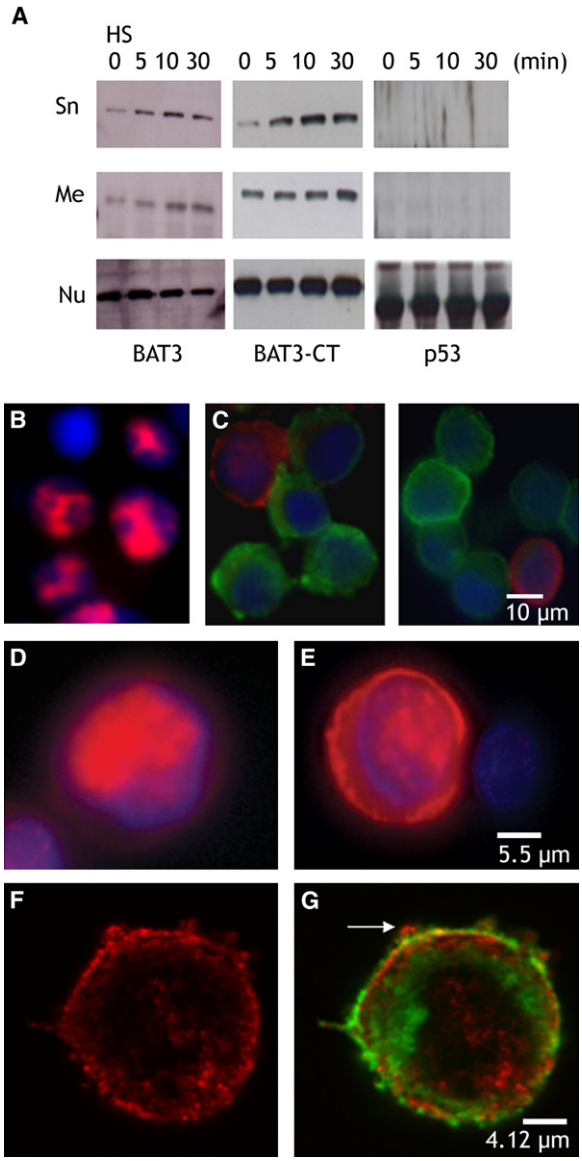

Figure 2. Expression Pattern of BAT3 in 293T Cells

(A) Immunoblotting to detect BAT3 or p53 in protein extracts (10 μg/lane) derived from supernatant (sn), membrane (me), or nucleus (nu) of 293T cells, that were left untreated (0 min HS) or exposed to a heat shock for the indicated time (min HS, minutes heat shock). Left, endogenous BAT3; middle, transfected BAT3-CT; right, p53 blot. See Figures S2A and S2B for the BAT3 antiserum (rabbit).

(B–E) $(His)_6$BAT3-transfected 293T cells were grown on coverslips, left untreated (B, D), or incubated for 2 hr with NKL cells or primary NK cells (C, E) in the ratio of 1:20 and stained after acetone fixation with a monoclonal his antibody and a-mouse-cy3-labeled secondary antibody (red). (C) shows two examples for a costaining of NKL cells with a CD30 antibody (anti-CD30-FITC, green), which specifically recognizes the CD30 surface receptor expressed on NK cells. Cell nuclei were stained with DAPI (blue). Pictures were acquired with the digital Nikon Eclipse E800 microscope with the LuciaGF program (Nikon, Düsseldorf, Germany) with a 10× NA 0.17 (B, C) or a 60× NA 0.23 objective (D, E) and processed with Photoshop software (Adobe).

(F and G) Cytospins of $(His)_6$BAT3-transfected 293T were stained after acetone fixation with a-His-cy3 (a-his) to detect BAT3 (red [F]) and with FITC-labeled choleratoxin (Ctx, green), which stains the plasma membrane of 293T cells (merge [G]). Fluorescence images were acquired by confocal laser scanning microscopy (Leica) with oil objective at pinhole size Airy 1. Crosstalk was minimized by serial acquisition of the fluorescence color channels. The digital images were merged by Leica LSM software without further processing. 100× objective lens; NA 1,4; FITC and cy3 were excited by 488 and 543 nm laser light and emission was detected at 495–530 nm and 548–700 nm, respectively.

surface expression and/or release of BAT3 are a prerequisite for a direct interaction with NKp30 and can thus be regarded as a part of tumor cells-NK cell interaction that leads to NK cell activation and tumor cell lysis. There is growing evidence that NK cell activation is a two-stage process, and it was recently demonstrated that NK cell/tumor cell cocultivation is a crucial step for the activation ("priming") of natural cytotoxicity (North et al., 2007).

To confirm membrane expression, we stained the plasma membrane with FITC-labeled choleratoxin (FITC-CtxB). FITC-CtxB specifically stains the lipid ganglioside GM1 predominantly anchored in the outer leaflet of lipid raft domains (Harder et al., 1998). Confocal microscopy scanning revealed expression of BAT3 (Figure 2F, red) in the plasma membrane compartment, because BAT3 and GM1 appeared partially colocalized (Figure 2G, yellow). A similar change in the expression pattern of BAT3 was observed upon exposure to a nonlethal heat shock, whereas the distribution of a nuclear control protein ($(His)_6$HNF4) remained unchanged (Figure S2D). Taken together, these results provide evidence that BAT3 is released from living cells in an inducible manner. BAT3 is therefore at least transiently accessible for a direct interaction with NKp30 expressed on NK cells.

Direct Interaction between Tumor Cell-Derived BAT3 and NKp30

We next performed experiments to substantiate the claim that BAT3 was the natural ligand for NKp30. Because a direct binding to NKp30 is a prerequisite for a possible biological function of released BAT3, the binding properties of BAT3-enriched supernatant and purified recombinant BAT3 protein were tested. BAT3 released from tumor cells is, in contrast to the purified soluble protein, predominantly detectable in the membrane vesicle fraction (Figures 3A and 3B) also containing HSP70 and the exosomal marker LAMP2b.

The in vitro binding of BAT3 to NKp30 was demonstrated with a BAT3-specific ELISA. A specific binding to immobilized NKp30-Ig, but not to NKG2D-Ig and CD30-Ig, was observed for supernatant derived from BAT3-transfected cells (Figure 3C, black bars) compared to control supernatant from wild-type cells (Figure 3C, white bars). The ELISA revealed also a specific binding of purified BAT3 derived from BAT3-transfected cells (Figure 3D, black bars) versus control purifications of wild-type cells (Figure 3D, white bars) to NKp30-Ig. The purified protein shows also a weak binding to NKG2D-Ig. Because a direct NKG2D-BAT3 interaction was not observed in

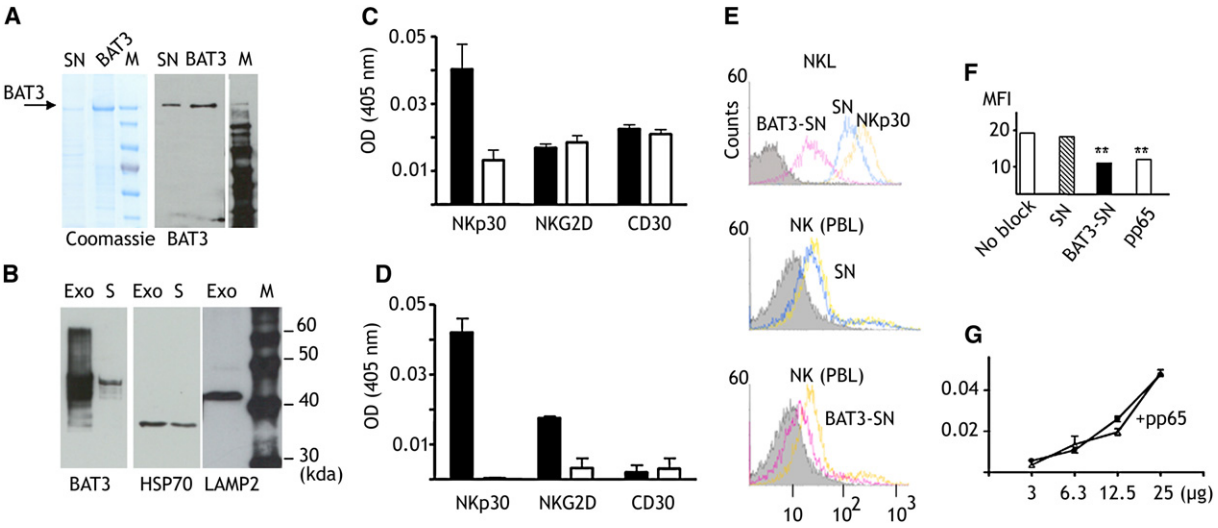

Figure 3. Interaction of Tumor Cell-Derived BAT3 with NKp30 on NK Cells

(A) Coomassie staining and immunoblotting of supernatants (SN) and affinity-purified protein (BAT3) derived from BAT3-transfected 293T cells. Loading: 20 μl of a 5-fold concentrated SN and 200 ng of the purified protein for coomassie staining and a 1:10 dilution for immunoblotting. The coomassie marker (m, lane 3) corresponds to 170 kda, 130 kda, 95 kda, 72 kda, 55 kda, 43 kda.

(B) The supernatant was fractionated by ultracentrifugation to detect BAT3, HSP70, and the exosomal marker LAMP2b in the membrane vesicles and soluble fraction by immunoblotting (10 μg loaded in each lane).

(C and D) ELISA plates were coated with recombinant NKp30-Ig, NKG2D-Ig, or CD30-Ig (concentration 100 ng/well) followed by incubation with 100 μl BAT3-enriched supernatant from BAT3-transfected 293T cells (black bars), control supernatant from wild-type cells (white bars) (C) or with 5 μg purified BAT3 protein (black bars), or with control purification from wild-type cells (white bars) (D). Binding was detected with anti-BAT3 serum and anti-rabbit-enzyme linked secondary antibody. Data represent absorbance at 405 nm after normalization to background of nonspecific binding to the plate. Error bars represent mean and standard deviation (SD) of triplicate samples.

(E) FACS analysis to demonstrate the effect of BAT3-SN on NKp30 mab binding to the human NK line NKL (top histogram) and to fresh NK cells isolated from peripheral blood lymphocytes (NK [PBL]) (middle and bottom histogram). NKL cells and NK (PBL) were left untreated or preincubated with either control supernatant (SN) or supernatant of BAT3-transfected 293T cells (BAT3-SN) as indicated. Cells were stained with mAb to NKp30. Gray histograms, background secondary antibody staining; yellow, untreated NK cells; blue, cells blocked with SN; pink, cells blocked with BAT3-SN.

(F) The effect of BAT3-SN on anti-NKp30 binding to fresh NK cells bars indicate the mean fluorescence intensity (MFI). The BAT3-SN- and pp65-mediated inhibition (presented for fresh NK cells) is significant ($p = 0.012$; $p = 0.042$, respectively; unpaired t test; GraphPadPrism software).

(G) ELISA plates were coated with recombinant NKp30-Ig (concentration 100 ng/well) followed by incubation with BSA (20 μg, filled triangle) or pp65 (20 μg, open triangle). All samples were incubated with recombinant BAT3 (3–25 μg), and binding of BAT3 was detected as described.

FACS experiments (not shown), we anticipate that the binding reflects unspecific protein interactions or might be attributed to traces of other soluble factors that were copurified with BAT3.

We also tested whether tumor-cell derived BAT3 (BAT3-enriched supernatant [BAT3-SN]) was able to bind to NKp30 on the surface of NK cells. Preincubation of NKL cells with BAT3-SN, but not with a control supernatant (Figure 3E, top), blocked the binding of anti-NKp30 (the preincubation did not interfere with the binding of anti-NKG2D; not shown). The reproducible inhibition of NKp30 binding with BAT3-SN was even observed with fresh NK cells isolated from peripheral blood lymphocytes that have a low expression of NKp30 (Figure 3E).

Similar binding activity to NKp30 on NK cells was recently reported for pp65, the viral NKp30 ligand (Arnon et al., 2005). In agreement, the inhibition of anti-NKp30 binding here presented for fresh NK cells was observed for BAT3-SN and pp65 (Figure 3F). However, blocking of NKp30 by pp65 did not interfere with BAT3 binding to plate-bound NKp30-Ig (Figure 3G), suggesting that BAT3 and pp65 bind to different, not overlapping, NKp30 domains.

These binding studies demonstrated that tumor cell-derived BAT3 was able to bind to NKp30, and we subsequently analyzed the functional consequences of this interaction.

BAT3-Mediated Cytokine Release

The secretion of IFN-γ and TNF-α from NK cells is crucial for the reciprocal activation of NK and dendritic cells and can be mediated through NKp30 (Ferlazzo et al., 2002; Pende et al., 1999; Vitale et al., 2005). Therefore, we analyzed the influence of BAT3 on the NK cell-dependent cytokine release (IFN-γ, TNF-α). NK cells were incubated for 2 days with medium (med), control supernatant (mock), or supernatant containing BAT3 (BAT3-SN). The cytokine secretion was stimulated with BAT3-SN, and the stimulation could be blocked with NKp30- and BAT3-specific antibodies (Figure 4A, left). The blocking was not always complete, particularly for the BAT3 antibodies, suggesting that factors distinct from BAT3 may also contribute to the NK

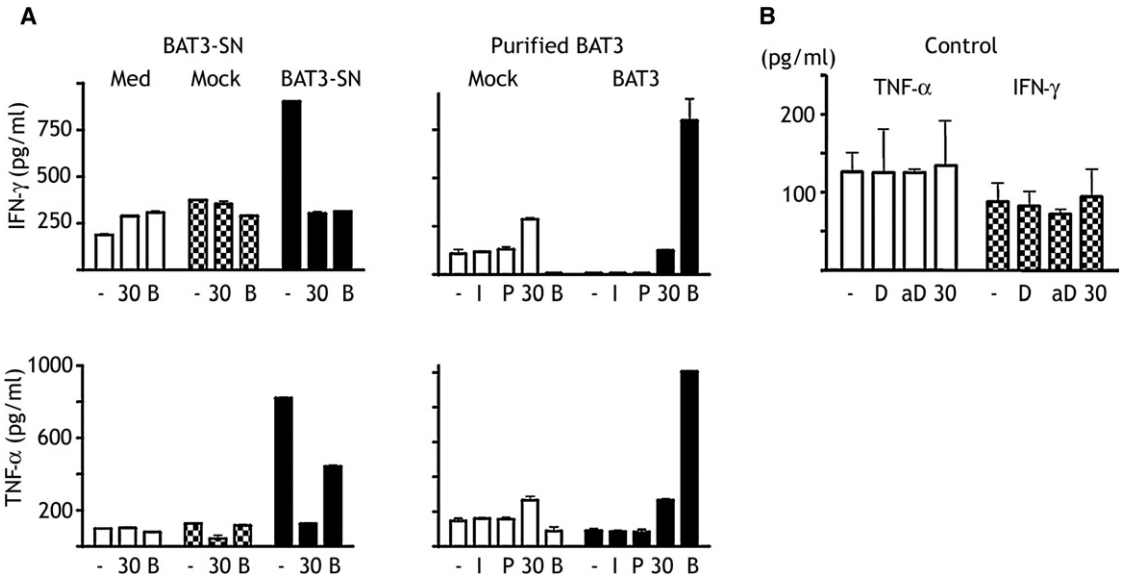

Figure 4. NK Cell-Dependent Cytokine Release in Response to BAT3

(A) Primary NK cells were incubated for 48 hr with medium (med), supernatant derived either from vector-transfected cells (mock) or from BAT3-transfected 293T cells (BAT3-SN) (left) or with purified protein derived from mock or BAT3-SN, respectively (right). The following antibodies were added as indicated: 30, NKp30; B, BAT3 (left, chicken; right, rabbit); I, NKp30 isotype; P, rabbit preimmune serum. The NK cell-derived supernatants were analyzed for IFN-γ and TNF-α content by specific ELISA. The means of triplicates and SD (pg/ml) are indicated. One representative experiment of three is shown.

(B) Primary NK cells were incubated with medium (-) or with a purified control protein DCoH (D) alone and in combination with a DCoH-specific antiserum (aD) or NKp30 (30), and the supernatants were analyzed for TNF-α and IFN-γ.

cell activation. Apparently paradoxically, the purified protein failed to induce any cytokine secretion per se, but revealed rather an inhibitory effect. Here the coincubation with BAT3-specific antibodies, which may alter the conformation or crosslink-purified recombinant BAT3, resulted in a strong cytokine release (Figure 4A, right). Control experiments with a purified soluble antigen and a specific rabbit antiserum revealed that the activation was not mediated via antigen-antibody complexes, e.g., via CD16 (Figure 4B). Thus, we demonstrated that BAT3 released from tumor cells (e.g., in membrane vesicles such as exosomes) activated NK cells via engaging NKp30, whereas the purified soluble protein inhibited NK cell-dependent cytokine release.

Effects of BAT3 Downregulation and BAT3 Overexpression on NK Cell-Dependent Lysis

Third, loss- and gain-of-function experiments were performed to investigate the functional properties of BAT3. A reduction of the BAT3 mRNA and protein amounts was achieved after transfection of HeLa cells with the corresponding siRNA (Figure 5A). The siRNA-transfected cells were less efficiently lysed by peripheral blood-derived NK cells than the control transfected cells (Figure 5B). The decrease of NK cell-mediated lysis was reproducible with different donors, although the degree of inhibition varied. This reflects most likely the varying impact of triggering receptors and their ligands including NKp30, NKp46, and others depending on their expression profile. Interestingly, the blocking of NKp30-dependent lysis by a masking antibody was less efficient than BAT3 downregulation, particularly when the effector:target ratio was high (Figure 5B). We speculate that this might be related to a direct or indirect BAT3-dependent engagement of triggering receptors distinct from NKp30. Vice versa, blocking by anti-NKp30 had still minor effects on NK cell-mediated cytotoxicity of BAT3-siRNA HeLa target cells. Remaining NKp30-mediated lysis upon BAT3 downregulation is not surprising, because siRNA downregulation of BAT3 is not complete (see Figure 5A). In addition, it cannot be excluded that tumor cells express or coexpress distinct ligands for NKp30, either on the cell surface or released, that may interfere.

The overexpression of BAT3 in the colon carcinoma cell line LS174T induced an enhanced NK cell-mediated lysis (Figure 5C). This effect was NKp30 mediated, because it was blocked by preincubating the target cells with the NKp30-Ig fusion protein in order to block NKp30 ligands. Taken together, these results suggest that BAT3 is an activating ligand for NKp30.

BAT3 Is Crucial for NK Cell-Mediated Lysis In Vitro and In Vivo

Finally, the function of endogenous BAT3 was addressed in vitro and in vivo. BAT3 antibodies that were able to effectively deplete BAT3 from the cell supernatant (Figure 6A) were used to inhibit the NKp30-dependent lysis of Raji cells (Figure 6B). Best results were obtained with

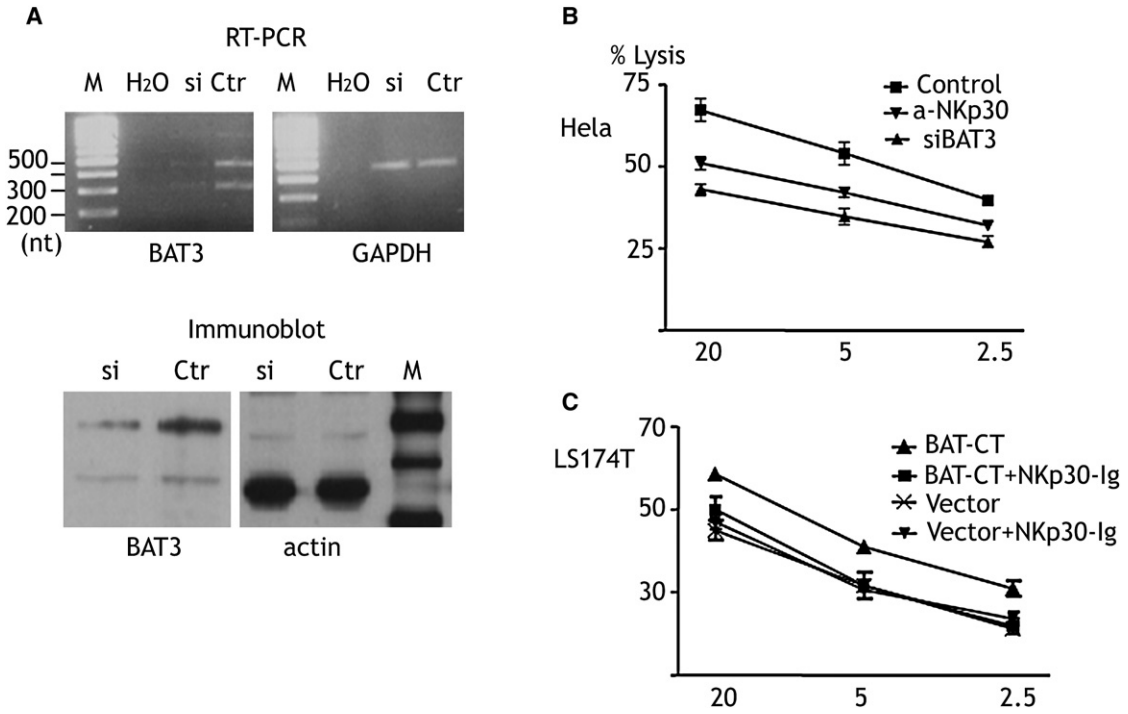

Figure 5. BAT3-NKp30 Mediated NK Cell Cytotoxicity

(A) Downregulation of BAT3 expression in HeLa cells. HeLa cells were transfected with control siRNA or BAT3-siRNA, and mRNA was isolated and analyzed for BAT3 and GAPDH expression by RT-PCR with gene-specific primers. BAT3-specific primers flank the BAG domain, and isoforms either lacking or including the BAG domain were detected (PCR products: 311 nt and 450 nt, respectively). Total protein lysates were prepared and analyzed for BAT3 and actin expression by specific immunoblotting. m, molecular weight marker; H_2O, control lacking cDNA; si, BAT3-siRNA; ctr, control.

(B) NK cell-mediated lysis of HeLa cells. HeLa cells transfected either with si control RNA or with BAT3-siRNA were incubated with primary NK cells at different effector:target ratios, and the lysis of the target cells was determined in an europium release assay. An isotype and an NKp30 antibody were used to block lysis of the target cells, and the NKp30-mab dependent lysis inhibition of BAT3-wild-type cells is indicated. The decrease of cell lysis upon BAT3 downregulation is significant ($p = 0.0063$; paired t test, one tailed, GraphPadPrism software).

(C) Primary NK cell lysis of LS174T cells transfected with vector or BAT3-CT. LS174T cells were transfected with a control vector or a BAT3 expression vector. The target cells were preincubated with NKp30-Ig (15 $\mu g\ ml^{-1}$) prior lysis to block NKp30 ligands as indicated. Effector and target cells were coincubated at different effector:target ratios. The increase of cell lysis is significant for BAT3-CT-transfected cells compared to vector-transfected cells ($p = 0.0074$), only if NKp30 ligands are not blocked with NKp30-Ig (paired t test, one-tailed, GraphPadPrism software). Error bars represent mean ± SD of triplicate samples. CD30-Ig used as a control did not alter sensitivity of vector- or BAT3-CT-transfected cells and no differences in lysis were observed for untransfected versus vector-transfected target cells (not shown). One representative experiment of three is shown (B, C).

an unusual long incubation period for the europium release assay (14 hr). This has also been described for the inhibition of NKp30 lysis by its viral ligand pp65 (Arnon et al., 2005).

Subsequently, we monitored the growth of RPMI8226-derived tumors in nude mice in the presence of human peripheral blood lymphocytes (PBL), either with a control antiserum (rabbit) or with blocking BAT3-specific antiserum (Figure 6C). The rapid growth of subcutaneous tumors (8/10) could be suppressed completely by treatment with human PBLs (0/10). In contrast, the simultaneous injection of BAT3-specific antibodies resulted in a decreased tumor rejection (6/10), indicating that BAT3 is crucial for tumor cell recognition and killing in this model.

DISCUSSION

In this study, we have shown that BAT3 may serve as a cellular ligand for NKp30 because (1) BAT3 binds directly to NKp30, (2) BAT3 inhibition or overexpression reduces or enhances NK cell-mediated killing, respectively, (3) released BAT3 triggers cytokine secretion of NK cells (TNF-α, IFN-γ), and (4) BAT3 mediates tumor rejection in a multiple myeloma xenograft model.

BAT3 is structurally characterized by C-terminal nuclear localization signals, an N-terminal ubiquitin-like region, a polyproline stretch, and the conserved BAG (Bcl-associated anthogene) domain that interacts with HSP70 (Manchen and Hubberstey, 2001; Takayama et al., 1997; Thress et al., 2001). Thus, BAT3, as a nuclear factor, is not reminiscent of a classical ligand. The protein also lacks any leader sequences targeting it to the classical secretion pathways. The data presented here demonstrate that BAT3 is released from tumor cells and may then interact with NKp30 on the surface of NK cells.

The possibility that NKp30 might have intracellular ligands was raised for the first time by Arnon et al. (2005). They recently identified the viral NKp30 ligand pp65 from

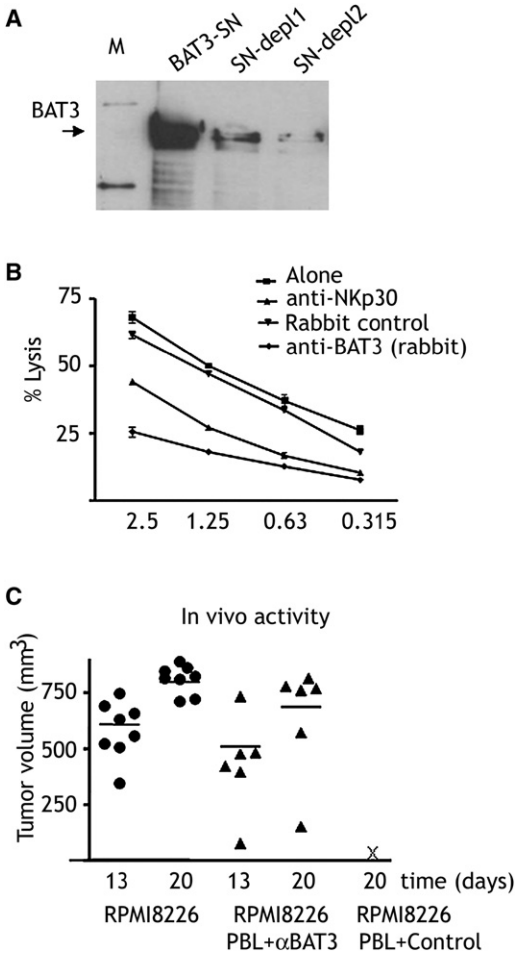

Figure 6. In Vivo Activity of BAT3

(A) Two BAT3-specific rabbit antisera were used in a 1:5000 dilution to deplete BAT3 from 293T-derived supernatants. SN-depl2 was chosen for further experiments.

(B) Raji cells were incubated with primary NK cells for 14 hr at different effector:target ratios without competing antibodies (alone) or with an rabbit preimmune serum (rabbit control), anti-NKp30, and anti-BAT3. The percent lysis was determined in a europium release assay.

(C) CD1 nude mice received 5 × 10^6 RPMI8226 cells subcutanously alone (RPMI8226) or in combination with 5 × 10^6 human peripheral blood lymphocytes that were mixed 5:1 with either 40 μl control rabbit antiserum (RPMI8226+PBL+ control) or 40 μl BAT3-specific antiserum (RPMI8226+PBL+αBAT3). The tumor volume at day 13 and 20 for each animal is indicated. Data from two independent experiments with five animals in each group are shown. The difference between RPMI8226+PBL+control versus RPMI8226+PBL+αBAT is significant ($p = 0.0168$, day 20) (paired t test, one tailed, GraphPadPrism software).

the human cytomegalovirus (CMV). This ligand induces a general suppression of the NK cell activity through a specific and direct interaction with NKp30 (Arnon et al., 2005). The viral factor pp65 has no structural homology to BAT3, but is like BAT3 localized in the cell nuclei (of infected cells). Therefore, it is unclear where and when pp65 interacts with NKp30 in vivo, and it has been speculated that soluble pp65 derived from the direct lysis of virus-infected cells or from apoptotic cells may bind to NKp30 (Arnon et al., 2005). However, the data presented here suggest a different mode of action for the release of the cellular ligand. We assume that active mechanisms, e.g., a modification of BAT3, might be involved, because BAT3 release from living cells was shown.

The BAT3-binding protein HSP70 is known as a potent stimulator that triggers the activity of NK cells, when expressed on the cell surface or upon secretion (Asea et al., 2000; Gastpar et al., 2005; Gehrmann et al., 2005; Millar et al., 2003). Although the mechanisms underlying the release of BAT3 remain to be elucidated, it is tempting to speculate that the transport is associated with exosomes, as described for HSP70 (Gastpar et al., 2005; Radons and Multhoff, 2005). In fact, a high molecular protein complex containing BAT3 and HSP70 was purified from 293T cells (unpublished data), and BAT3 was detectable in the membrane vesicles fraction of 293T-derived supernatant.

An exosomal release of BAT3 would also explain why BAT3-enriched supernatant derived from tumor cells stimulated the NK cell-mediated cytokine release, whereas the purified, recombinant protein acted in a rather suppressive manner. Opposite biological effects for membrane-expressed versus soluble factors is not an unusual observation for ligands engaging a triggering NK cell receptor. Surface expression of MICA (major histocompatibility complex class I chain-related gene A), a NKG2D-specific ligand, marks transformed cells for destruction by immune effector cells, whereas soluble ligands directly inhibit NKG2D-mediated activation and may promote tumor cell escape from immunosurveillance (Groh et al., 2002; Bottino et al., 2005).

Our functional in vivo findings on BAT3 were reminiscent to heat shock proteins. HSP70, a multifunctional factor acting as a chaperone or cytokine, can induce tumor rejection when purified from the tumor (Calderwood et al., 2005; Millar et al., 2003). Interestingly, it has been reported that nuclear protein complexes that contain HSPc70 together with high mobility group (HMG) proteins B1 and B2 and with BAT3 and HSPc70 are involved in the cytotoxic response to DNA damage (Krynetski et al., 2003). HMGB1 is, so far, the best characterized nuclear factor, which exhibits cytokine activity upon release, and this factor is mainly involved in NK-cell mediated maturation of dendritic cells (Lotze and Tracey, 2005; Semino et al., 2005). HMGB1 is thought to transduce its function through Toll-like receptors (TLR 2 and 4) and receptor of advanced glycation end products (RAGE), although other not-yet-identified receptors might be involved.

It is conceivable that such nuclear proteins, which exhibit cytokine function to alert the immune system, are sensors for DNA instability or damage of transformed cells. This model is supported by the exciting finding that BAT3 is crucial for the p53 acetylation (Sasaki et al., 2007), a central event for the cellular DNA damage response. A direct link of the DNA damage response to innate immunity and cancer was recently discovered by Gasser et al. (2005), demonstrating that the DNA damage

pathway directly regulated the expression of ligands engaging the NKG2D receptor.

Factors such as HSP70, HMGB1, and BAT3 are emerging as a class of immune regulatory proteins, which may be regarded as an intracellular subgroup of a larger set, the damage-associated molecular patterns (DAMPs) (see recent review by Bianchi [2007]).

The data presented here show that a released factor engages a triggering receptor on NK cells and thus support a model for a ''damage-induced recognition'' (Matzinger, 2002) by factors released from tumor cells or accessory cells (dendritic cells, macrophages).

EXPERIMENTAL PROCEDURES

Yeast Two-Hybrid Screening

We used a K562-derived cDNA library (BD Clontech, Germany, Heidelberg [HL4032AH]) and the yeast expression plasmids pGBT9 and pGADT7 (BD Clontech) to identify NKp30 interaction partners. The DNA encoding the NKp30 leader and extracellular domain (amino acids 1–110, accession number gi 24475831) was amplified by the polymerase chain reaction (PCR) from cDNA from the human NK line NKL (Robertson et al., 1996). The PCR-generated fragment was cloned into the yeast expression vector pGBT9 in-frame with the GAL4-DNA-binding domain. The clones interacting with NKp30 were sequenced and a gene bank search (NCBI blast) revealed the isolation of BAT3-CT (accession number gi 149158691). The BAT3 cDNAs either including or lacking the BAG domain were amplified by PCR from K562-derived cDNA, cloned into pGADT7, and tested for NKp30 interaction. The BAT3 deletion constructs were generated either by PCR amplification or by means of internal restriction sites. The DNA fragment encoding NKp46 (amino acids 1–287, accession number gi 89363035) was amplified by PCR from NKL-derived cDNA and cloned into pGBT9.

RT-PCR

BAT3 transcripts were amplified by RT-PCR with sequence-specific primers (5′TTCCCCAGCCCCTGGAA and 5′TTGGGGAAACCCTGGG GACTGT). The human tumor cDNA panel for the PCR was obtained by BD, Clontech (pt3158).

Cells

We used the following cell lines: the human fibroblast kidney cell line 293T, the human colon carcinoma cell line LS174T, the human multiple myeloma cell line RPMI8226, and the human NK cell line NKL (Robertson et al., 1996). NK cells were obtained from peripheral blood mononuclear cells (PBMCs) from healthy-donor buffy coats by Ficoll-Paque density gradient centrifugation with Leucosep columns from Greiner bio-one (Solingen, Germany). Non-NK cells were depleted with the NK Cell Isolation Kit and VarioMACS (Miltenyi, Bergisch Gladbach, Germany). Separated polyclonal NK cells were cultivated in minimal essential medium (MEM) alpha supplemented with 50 μg/mL penicillin, 50 μg/mL streptomycin, 10% fetal calf serum, and 10 U/mL recombinant human IL-2 (R&D Systems, Wiesbaden, Germany) at 37°C with 5% CO_2. The cytokine release assay (Figure 4) was performed without addition of IL-2.

Primary immature dendritic cells (iDCs) were obtained from adherend peripheral blood monocytes after incubation for 5 days in the presence of IL-4 and GM-CSF (R&D Systems, Wiesbaden Germany) at the final concentrations of 20 ng ml^{-1} and 50 ng ml^{-1}, respectively.

NKp30-Ig, CD30-Ig, and NKG2D-Ig Fusion Proteins

The sequence encoding the extracellular domain of NKp30 was amplified by PCR from NKL-derived cDNA and cloned into the expression vector pCDNA3.1 (Invitrogen, Karlsruhe, Germany), which contained the human IgG1 kappa leader sequence (N-terminal) and the genomic DNA of the Fc portion of human IgG1 (C-terminal). The CD30 extracellular sequence was inserted in-frame between the IgG1 kappa leader and the genomic DNA of the Fc portion of human IgG1. NKp30-Ig and CD30-Ig fusion proteins were purified from the supernatants of transfected 293T cells with protein-G sepharose beads as recommended by the manufacturer (Amersham Bioscience, Freiburg, Germany). Recombinant NKG2D-Ig was purchased from R&D Systems, Wiesbaden, Germany.

Antibodies

We used the following primary antibodies: anti-NKp30 (sc-20477, Santa Cruz Biotechnology, Heidelberg, Germany) for immunoblots; anti-NKp30 (mAb1849, R&D Systems, Wiesbaden, Germany) for FACS and blocking experiments; BH1-2A as anti-NKp30 isotype control (kindly provided by H. Stein, UK Berlin); anti-His (34660, QIAGEN, Hilden, Germany); anti-HSP70 (SPA810, Biomol, Hamburg, Germany); anti-LAMP2b (ab18529, Abcam, Germany); anti-BAT3 (ab37751, Abcam); and the monoclonal CD30 antibody 5F11 (Borchmann et al., 2003). The generation of the BAT3-specific rabbit antiserum (raised against the COOH-terminal region as an antigen (NH2)-RKVKPQPPLS DAYLSGMPAK) was described elsewhere (Desmots et al., 2005). The FITC-labeled choleratoxin (c-1655) was purchased from Sigma-Aldrich, Taufkirchen, Germany. The secondary antibodies were obtained from Dianova GmbH, Hamburg, Germany.

BAT3 Mammalian Expression Constructs

DNA fragments encoding BAT3-CT and BAT3 were cloned into the expression vectors pCDNA3.1 and pCDNA3.1-His (Invitrogen, Karlsruhe, Germany) in-frame with a C-terminal 6xhistidine tag $(His)_6$ and into pEGFP-C1 (BD Biosciences Clontech, Palo Alto, CA) in-frame with the N-terminal eGFP sequence.

Immunoprecipitation and BAT3 Depletion

The expression constructs encoding histidine-tagged BAT3 and BAT3-CT were transfected with lipofectamine (Invitrogen, Karlsruhe, Germany) into 293T wild-type cells and 293T cells constitutively expressing NKp30-Ig. Cell lysates were prepared after 48 hr with lysis buffer (50 mM Tris, 150 mM NaCl, 0.1% Triton X-100, 0.5% Sodium deoxcholate, 2 mM EDTA, and 0.1% SDS) containing cocktail protease inhibitors (Amersham Bioscience), and precipitation was performed with protein-G sepharose beads slurry (Amersham Bioscience) as recommended. The eluates were used for immunoblotting with His and Ig antibodies to detect His-tagged BAT3 proteins and NKp30-Ig, respectively.

For depletion of BAT3, cells were harvested exposed to a nonlethal heat shock and the supernatant was collected and incubated with two rabbit polyclonal sera raised against BAT3 (serum 25 and 23; Desmots et al., 2005) for 1 hr followed by incubation with protein-A beads over night (buffering conditions were maintained with 10× Tris-Nacl). The supernatant was used for immunoblotting to detect BAT3.

Immunofluorescence

The transfected target cells were cultured on glass slides and incubated with NK cells (ratio 1:20) as indicated. The cells were fixed with acetone before staining with monoclonal his antibody (QIAGEN), monoclonal CD30 antibody (Borchmann et al., 2003), or FITC-labeled choleratoxin (Sigma-Aldrich) followed by staining with labeled secondary antibodies. The antibodies were diluted in phosphate-buffered saline with 10% serum.

Tumor Cell-Derived Supernatant and Purified BAT3

BAT3-transfected 293T cells were either left untreated or exposed to a nonlethal heat shock. For the heat shock, 2 × 10^6 cells (eppendorf tube) were incubated with 1 ml medium at 42°C for 30 min followed by a recovery period at 37°C for 2 hr (BAT3-SN). BAT3 was purified with NiNTA affinity chromatography according to QIAGEN, Hilden, Germany (The QIAexpressionist, 2003) and dialyzed against PBS. Membrane vesicles were prepared from the supernatant by three

successive centrifugations at 300 × g (5 min), 1200 × g (20 min), and 10,000 × g (30 min) to eliminate cells and debris, followed by centrifugation for 1 hr at 100,000 × g. The exosome pellet was washed once in a large volume of PBS, centrifuged at 100,000 × g for 1 hr, and resuspended in PBS.

Expression and purification of the histidine-tagged control protein DCoH is described elsewhere (Pogge von Strandmann et al., 2000).

Cytotoxicity Assays

The cytotoxicity was estimated in a standard 5 hr europium release assay in a 96-well micro titer plate in a total volume of 200 μl with 5×10^3 tumor target cells and different effector:target ratios. NK cells were incubated with 50% serum for 30 min at 4°C, washed, and incubated with the monoclonal anti-NKp30 (R&D Systems, mAb1849) in the final concentration of 10 μg ml^{-1} for 1 hr at 4°C to block NKp30. Blocking of NKp30-Ig to BAT3-transfected cells was performed by incubating the target cells with 15 μg ml^{-1} NKp30-Ig for 30 min prior lysis. The blocking anti-BAT3 serum (rabbit) was used in a 1:1000 dilution. The spontaneous release did not exceed 25% of the maximum release.

Flow Cytometry

The binding of tumor cell-derived supernatant and anti-NKp30 mAb was detected on primary NK cells. Cells were preincubated with supernatant for 1 hr at 4°C for blocking experiments. Binding of antibodies to viable cells was analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Heidelberg, Germany).

IFN-γ and TNF-α Assay

1–2 × 10^5 primary NK cells were incubated for 48 hr with medium or supernatants derived from 293T cells either vector (mock) or BAT3-transfected (BAT3-SN) and with purified BAT3 protein or purified DCoH control protein (Pogge von Strandmann et al., 2000). Blocking monoclonal antibodies were used at a final concentration of 10 μg/ml, and the rabbit or chicken antisera were used 1:1000 diluted. The NK cell-derived supernatants were analyzed (final concentration 1:5 diluted) with IFN-γ and TNF-α ELISA Detection Kits (Becton Dickenson, Heidelberg, Germany). The absorbance of the plates was measured with the ELISA reader μ-Quant (Bio-Tek, Bad Friedrichshall, Germany) in parallel with the measurement of the corresponding standards.

Multiple Myeloma Xenograft Model

RPMI8226 cells (5×10^6) resuspended in 200 μl PBS were injected subcutaneously into CD1 nude mice (Charles River, Sulzfeld, Germany) to establish tumors. Peripheral blood lymphocytes were isolated from healthy donors by Ficoll-Paque density gradient centrifugation with Leucosep columns, mixed with rabbit control serum or a BAT3-specific antiserum (rabbit, 1:20 dilution), and injected subcutaneously ((5×10^6)/200 μl per animal). The experiments were performed in accordance with the institutional and national regulations (permission: 50.203.2-K17, 4/02).

Statistics

The results of the NK cell-activation assays are indicated as means ± standard deviation. Significance was calculated with the GraphPad-Prism software (San Diego, CA) with the Student's t test.

Supplemental Data

Three figures are available at http://www.immunity.com/cgi/content/full/27/6/965/DC1/.

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